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Synthesis of Epstein-Barr Virus DNA In Vitro: Effects of Phosphonoacetic Acid, *N*-Ethylmaleimide, and ATP

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Nuclei from superinfected Raji cells synthesized Epstein-Barr Virus (EBV) DNA in vitro in the absence of cell DNA synthesis. The synthesis of EBV DNA in vitro was inhibited by phosphonoacetic acid and *N*-ethylmaleimide, and maximum synthesis was achieved in the absence of an ATP-regenerating system. Nuclei from mock-infected cells required an ATP-regenerating system for maximum DNA synthesis.

The human lymphoblastoid cell line Raji (9) carries 50 to 60 copies of the Epstein-Barr virus (EBV) genome (6), but it does not produce infectious virus. When Raji cells are superinfected with EBV produced by P3HR-1 cells (9), they undergo an abortive, lytic infection characterized by shutdown of host DNA synthesis, breakdown of cell DNA, and a burst of EBV DNA replication (5). This viral DNA has a molecular weight of 10^8 and has been isolated from the nucleus of superinfected cells 22 h after infection. It has also been established that nuclei isolated from superinfected Raji cells are capable of supporting synthesis of EBV DNA in vitro (J. E. Shaw et al., submitted for publication). Here we extend those in vitro studies and report the effects of ATP and of two inhibitors of DNA polymerases, phosphonoacetic acid (PAA) and *N*-ethylmaleimide (NEM), on the in vitro synthesis of EBV DNA.

When superinfected Raji cells were labeled in phosphate-free medium, they preferentially synthesized EBV DNA with a minimum of synthesis of host cell DNA (Y. Yajima and M. Nonoyama, Abstr. Proc. Am. Assoc. Cancer Res. 16:116, 1975; J. E. Shaw et al., submitted for publication). Because it was desirable to minimize the incorporation of label into cellular DNA in these studies, nuclei were removed from cells that had been in phosphate-free medium from 6 to 22 h postinfection. This treatment significantly reduced the amount of label incorporated by nuclei from mock-infected cells by as much as 80% when an ATP-regenerating system was included in the reaction mixture and by approximately 70% in its absence (Table 1). Under the same phosphate-free conditions, nuclei from superinfected cells incorporated 18 times more label in the presence of an ATP-regenerating system and 127 times more in its absence. Clearly maximum synthesis by nuclei from mock-infected cells was achieved in the presence of an ATP-regenerating system, whereas the reverse was true for nuclei from superinfected cells.

Synthesis by nuclei from superinfected cells ceased after about 10 min in the presence of the ATP-regenerating system, whereas in its absence synthesis proceeded linearly in time, and at a greater rate, for approximately 20 min (Fig. 1A). The contrary situation was observed in nuclei from mock-infected cells, where the absence of the ATP-regenerating system strongly depressed synthesis (Fig. 1B).

The above findings suggested that synthesis of EBV DNA in nuclei was stimulated by the absence of the ATP-regenerating system. Therefore, the products synthesized in vitro by nuclei from superinfected cells were analyzed by equilibrium density gradient centrifugation in CsCl to determine whether they were viral or cellular in origin. In the presence of the ATP-regenerating system, some cellular DNA (ρ = 1.699 g/cm^3) was synthesized, but the predominant product had the density of EBV DNA (ρ = 1.718 g/cm^3) (Fig. 2). It is possible that the label entering cellular DNA was due to the presence of uninfected cells. This is consistent with the observation that 70% or fewer of the cells were early antigen positive after superinfection. In the absence of the ATP-regenerating system (Fig. 2B), significantly more label was incorporated into viral DNA, whereas there was a slight decrease in the amount of label entering cellular DNA. It was thus apparent that the increase of incorporation of label from [^{3}H]TTP in the absence of the ATP-regenerating system (Table 1) reflected an increased rate of EBV

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TABLE 1. *DNA synthesis in isolated nuclei from infected and mock-infected Raji cells*[a]

Origin of nuclei	cpm incorporated/10^6 nuclei[b]	
	I	II
Mock-infected, regular medium	17,818	4,762
Mock-infected, phosphate-free medium	3,632	1,300
Superinfected, phosphate-free medium	64,430	165,000

[a] Nuclei were isolated 22 h postinfection according to the procedure of Benz and Strominger (1) from superinfected and mock-infected Raji cells that had been placed in phosphate-free minimum essential medium containing 2% phosphate-free fetal bovine serum between 6 and 22 h after infection. The complete standard reaction mixture for assay of DNA synthesis in vitro contained in a final volume of 200 μl: HEPES buffer (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid), 25 mM, pH 8; NaCl, 40 mM; $MgCl_2$, 5 mM; EGTA (ethylene glycol-bis-*N,N'*-tetraacetic acid), 5 mM; spermine, 0.15 mM; spermidine, 0.5 mM; sucrose, 120 mM; dextran 100, 1%; dithiothreitol, 1 mM; dATP, dCTP, dGTP, each 0.2 mM; [^{3}H]TTP, final specific activity, 5,000 cpm/pmol, 0.005 mM; 0.5×10^6 to 5×10^6 nuclei; and an ATP-regenerating system consisting of 6 mM ATP, 6 mM phosphoenolpyruvate, and 2.5 μg of pyruvate kinase per ml. Where indicated, these latter reagents were substituted by water. The assays were performed for 30 min at 37°C and stopped by adding 0.5 ml of cold 10% trichloroacetic acid containing 20 mM sodium pyrophosphate. Acid-precipitable material was collected on Whatman GF-C filters, washed twice with 5% acid containing 20 mM sodium pyrophosphate and once with 70% ethanol, and then dried and counted in toluene-based scintillation fluid. All assays were routinely done in triplicate.

[b] I, Standard reaction mixture, complete. II, Standard reaction mixture, ATP-regenerating system omitted.

DNA synthesis. Hence the change in relative proportions of cellular and viral DNA synthesized in vitro depended on the presence or absence of the ATP-regenerating system. Its absence enhanced viral DNA synthesis but not the synthesis of cellular DNA. A similar situation has been observed with the in vitro synthesis of herpes simplex and vaccina virus DNA (2). In these two systems, nuclei from mock-infected cells required ATP for in vitro DNA synthesis, which dropped 10-fold in its absence, whereas nuclei from herpesvirus- or vaccina virus-infected cells showed no requirement for ATP.

EBV DNA replication in superinfected Raji cells in vivo was reported to be inhibited by PAA (12) in concentrations that do not interfere with normal cell proliferation (7, 10). Further investigation with this drug showed that it strongly inhibited DNA synthesis in vitro (Table 2). Density profiles of DNA synthesized in vitro by nuclei from superinfected Raji cells in the presence of 10 and 100 μg of PAA per ml are shown in Fig. 3. These profiles indicated that in vitro the synthesis of both cellular and viral DNA was inhibited by PAA, although synthesis of cellular DNA may have been inhibited to a somewhat lesser degree than synthesis of EBV DNA. This inhibition of cellular DNA synthesis in vitro by PAA is at present difficult to explain with the demonstrated resistance of purified cellular DNA polymerases α, β, and γ from Raji cells to PAA concentrations up to 200 μg/ml (E. S. Huang, personal communication). However, it should be noted that the DNA synthesis observed in these studies may not be normal polymerase-mediated replicative synthesis.

When the sulfhydryl blocking agent NEM was included in the reaction mixture, in vitro DNA synthesis in nuclei from superinfected Raji cells was inhibited by more than 95% (Table 2). Analysis of the density profile of DNA

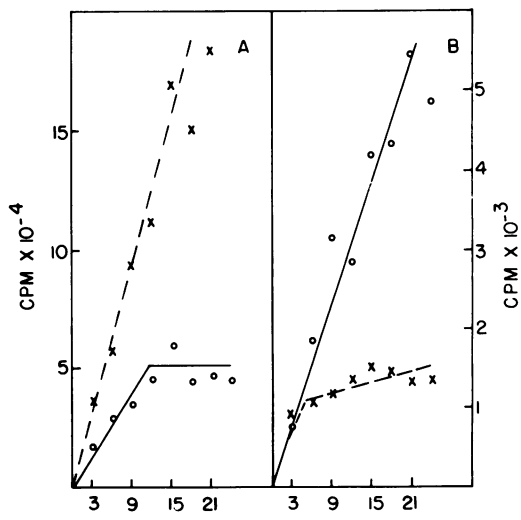

FIG. 1. *Kinetics of in vitro incorporation of label from [^{3}H]TTP in nuclei from superinfected and mock-infected Raji cells in the presence and absence of an ATP-regenerating system. Nuclei from superinfected (A) and mock-infected (B) cells were assayed in the standard reaction mixture at 37°C in the presence* (○) *or absence* (×) *of the ATP-regenerating system. At the time indicated, duplicate 10-μl aliquots were precipitated with trichloroacetic acid. Note the difference in scales between panels A and B. Each point represents incorporation of label by 10^6 nuclei.*

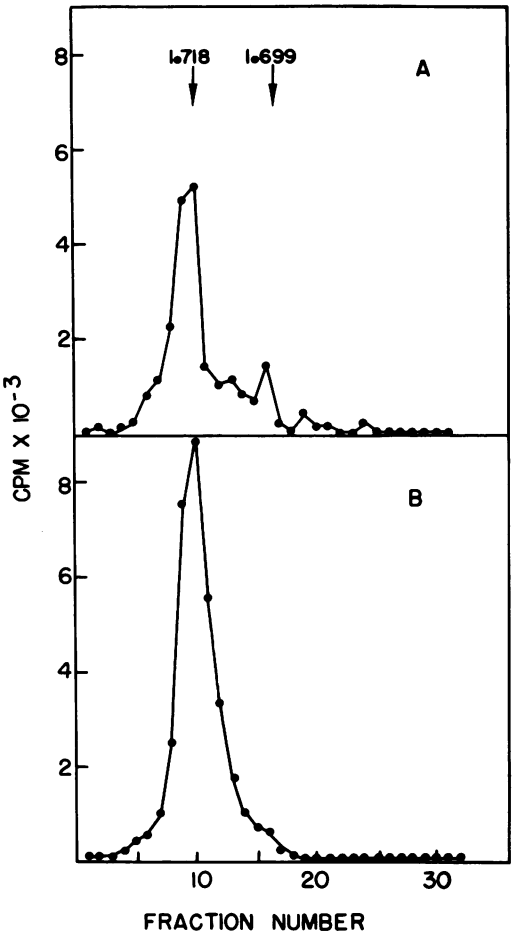

FIG. 2. *Density profile of DNA synthesized in vitro in nuclei from superinfected Raji cells in the presence (A) and absence (B) of the ATP-regenerating system. Nuclei from superinfected Raji cells were incubated in the standard reaction mixture for 30 min at 37°C. The reaction was stopped by the addition of 2 ml of cold TBS (NaCl, 150 mM; Tris-hydrochloride, pH 7.4, 50 mM), and nuclei were pelleted for 3 min at 1,400 × g and then resuspended in 0.5 ml of TBS containing 50 mM EDTA. They were lysed by adding 0.15 ml of 3% Sarkosyl-97 and digested for 3 h at 37°C with 100 μg of Pronase per ml. The lysate was then phenol extracted, and the DNA was precipitated with ethanol and dissolved in 4 ml of 0.1 SSC (0.15 M NaCl + 0.015 M sodium citrate). Solid CsCl (5 g) was added, and the DNA was centrifuged to equilibrium in a T-40 rotor (Spinco) at 35,000 rpm at 20°C for 60 to 70 h. Fractions (0.2 ml) were collected and acid precipitated onto Whatman GF-C filters. The arrows indicate the positions of ^{32}P-labeled EBV DNA (ρ = 1.718 g/cm³) and of unlabeled Raji DNA (ρ = 1.699 g/cm³) added as markers.*

made in vitro in the presence of 2 mM NEM shows that most of the residual incorporation was into cellular DNA (Fig. 4). This suggested that EBV-specific DNA polymerase was as sensitive to NEM as were the cellular DNA polymerases α and γ and that the observed residual incorporation of label into cellular DNA was probably mediated by the NEM-resistant cellular polymerase β (11).

Benz and Strominger (1) have demonstrated that nuclei from human lymphoblastoid cells supported the synthesis of EBV DNA in vitro. Most of the DNA replicated, however, except

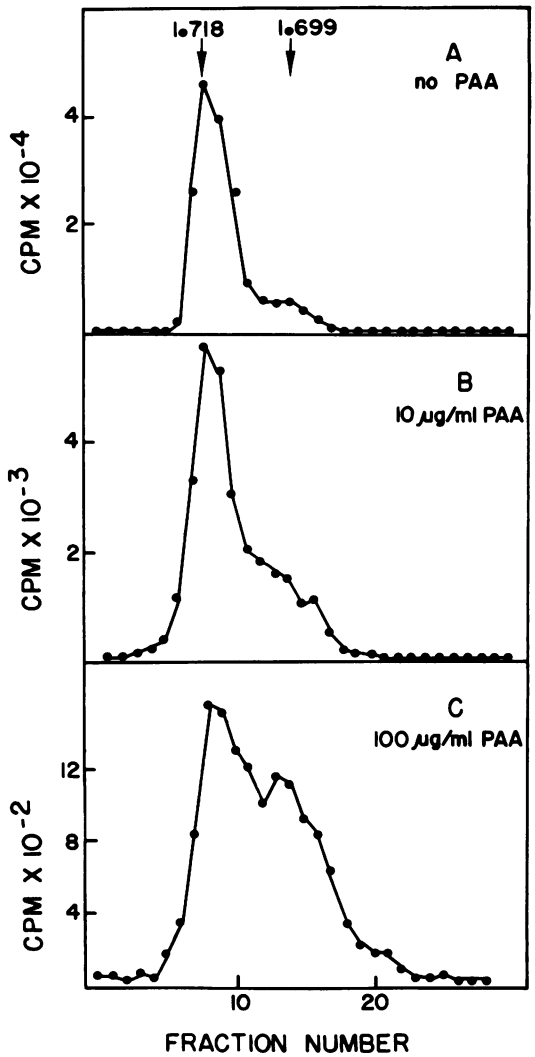

FIG. 3. *Effect of PAA on the in vitro synthesis of viral and cellular DNA. Nuclei from superinfected Raji cells were incubated for 30 min at 37°C in the complete standard reaction mixture containing 0, 10, or 100 μg of PAA per ml, respectively. DNA was then extracted, mixed with ^{32}P-labeled DNA marker, and centrifuged to equilibrium as described for Fig. 2. Note the different scales in the individual panels.*

TABLE 2. *Inhibition of in vitro DNA synthesis by phosphonoacetic acid (PAA) or N-ethylmaleimide (NEM) in nuclei from superinfected Raji cells*[a]

Concn of PAA (μg/ml) or NEM (mmol/liter)	cpm incorporated/10^6 nuclei	% Inhibition
0 PAA	47,300	
10 PAA	23,242	40.3
100 PAA	3,992	92.2
200 PAA	3,557	92.5
2.5 NEM	1,942	95.9

[a] Nuclei from superinfected Raji cells were assayed for 30 min at 37°C in the complete standard reaction mixture (see Table 1), containing PAA or NEM. The PAA stock solution was 2 mg/ml in 25 mM HEPES buffer adjusted to pH 7.8 with NaOH.

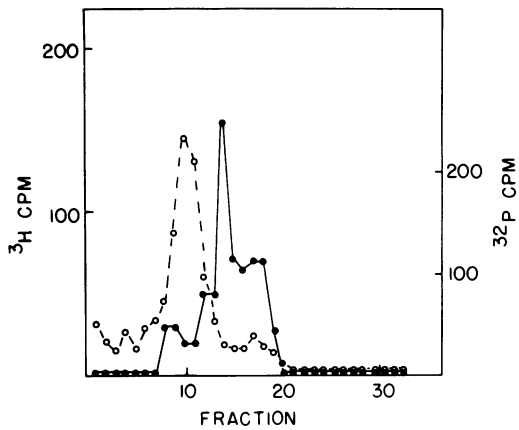

FIG. 4. *Effect of NEM on the in vitro synthesis of viral DNA. Nuclei from superinfected Raji cells were preincubated with 2 mM NEM in ice for 10 min and then kept for 30 min at 37°C in the complete standard reaction mixture, containing 2 mM NEM and lacking dithiothreitol. The nuclei were prepared for CsCl centrifugation as described in Fig. 2. Symbols: (●) ^{3}H-labeled in vitro DNA; (○) ^{32}P-labeled EBV DNA marker.*

during early S-phase, was that of lymphoblastoid cells. We have shown in this report that if cells from the lymphoblastoid line Raji were superinfected with EBV before nuclear isolation, most of the product made in vitro was EBV DNA. We have used this system to demonstrate that the synthesis of EBV DNA in vitro was sensitive to the action of NEM, a potent inhibitor of DNA polymerases, and to PAA, a drug that inhibited the replication of other herpesviruses (4; R. G. Duff and L. R. Overby, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, S161, p. 240) and herpesvirus DNA by nuclei in vitro (2, 3). Because EBV DNA was synthesized virtually in the absence of cellular DNA synthesis (Fig. 2B), this in vitro system should be useful for the direct evaluation of drugs suspected of interacting with the EBV-specific DNA polymerase and, as well, useful as a first step in the purification of a template-free viral enzyme.

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